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Tracing the location of powdery mildew resistance-related gene *Stpk-V* by FISH with a TAC clone in *Triticum aestivum–Haynaldia villosa* alien chromosome lines

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Bacterial artificial chromosomes (BACs) or yeast artificial chromosomes (YACs) containing large inserts as probes for fluorescence *in situ* hybridization (FISH) have been used in the physical mapping of specific DNA sequences, especially for single- or low-copy sequences. Our earlier study identified *Stpk-V*, a powdery mildew resistance-related gene located on the 6VS chromosome arm of the wild grass *Haynaldia villosa* (tribe *Triticeae*), and obtained several *Triticum aestivum–H. villosa* alien chromosome lines carrying the *Stpk-V* gene. However, the precise physical location of the *Stpk-V* gene on chromosome 6VS is not known. In this study, we used TAC-FISH with TAC15 as the probe coupled with sequential genomic *in situ* hybridization (GISH) to determine the physical location of the *Stpk-V* gene in different *T. aestivum–H. villosa* 6V alien chromosome lines, including addition, substitution and translocation lines. The result indicated that the fraction length of the *Stpk-V* locus is 0.575±0.035 on the 6V chromosome short arm and this was confirmed by FISH using TAC15 as the probe for tracing the *Stpk-V* gene in other genetic stocks. The cytological mapping strategies used in this study will be of benefit for tracing the alien gene location in the course of introducing desirable traits from wild species.

fluorescence *in situ* hybridization (FISH), transformation-competent artificial chromosome (TAC), serine/threonine protein kinase gene from the V-genome (*Stpk-V*), *Triticum aestivum–Haynaldia villosa* alien chromosome lines

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Fluorescence *in situ* hybridization (FISH) has been widely used for mapping repetitive DNA sequences and multi-copy gene families [1]. Genomic *in situ* hybridization (GISH), a modification of FISH using total genomic DNA from one species as the probe, is a powerful tool for the detection of introgressed alien chromatins [2]. In the tribe *Triticeae*, localized repetitive sequences are useful as cytogenetic markers for chromosome identification and GISH has been used for detecting alien chromosomes in wheat (*Triticum aestivum*) alien chromosomal lines [3,4]. The physical mapping of single- or low-copy sequences on metaphase chromosomes is difficult but necessary for gene or marker localization in wheat.

The use of genomic DNA fragments in large-insert vectors, such as bacterial artificial chromosomes (BACs) or yeast artificial chromosomes (YACs), in combination with FISH (BAC-FISH or YAC-FISH) has been exploited for mapping genes in some plant species with small genomes, including rice (*Oryza sativa*) [5−9], sorghum (*Sorghum* sp.) [10], *Ipomoea trifida* [11] and *Beta vulgaris* [12]. It is difficult to generate unique locus-specific FISH signals from most wheat BACs because the wheat genome contains a huge content of repetitive DNA sequences (tandem repeats, transposable elements) scattered throughout the genome [3]. In order to physically map a useful gene *Stpk-V* from the wild grass *Haynaldia villosa* (*Dasypyrum villosum,* 2*n*=2*x*= 14, genome VV), a positive transformation-competent artificial chromosome (TAC) clone containing the gene was

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used as the probe for FISH to try to determine the exact physical location of the gene in this study.

Powdery mildew (PM), caused by *Blumeria graminis* f. sp. *tritici*, is one of the major diseases of wheat worldwide. *Pm21,* a gene located in the 6VS chromosome bin (fragment length 0.00–0.58) of *H. villosa*, confers a high level of broad-spectrum resistance to *B. graminis* [13,14]. A serine/threonine kinase gene *Stpk-V* of *H. villosa*, which is up-regulated by *B. graminis* inoculation, was isolated using microarray in our earlier work, and the sequence-specific PCR marker CINAU15, which is linked to *Pm21*, was designed on the basis of the sequence of the *Stpk-V* gene [15]. The location of the *Stpk-V* gene was located on the 6VS chromosome by PCR analysis in materials containing 6VS, and further mapped to the chromosome bin FL 0.45–0.58 by using a resistant alien deletion line del6VS-1 (FL 0.58) and a susceptible alien deletion line del6VS-2 (FL 0.45) [15]. TAC15 and a 5160-bp sub-clone containing the whole genomic sequence of *Stpk-V* were obtained by screening a TAC library of T6VS/6AL with the gene-based marker CINAU15 [16,17]. Sequence comparisons showed that the cDNA of *Stpk-V* matches the exons of the TAC sub-clone completely. Therefore, TAC15 was suitable for physical mapping of the *Stpk-V* gene. A TAC-FISH signal was detected near the telomeres of 6VS in del6VS-1, and the signal was located from FL 0.45 to 0.58 of 6VS in the *T. aestivum–H. villosa* translocation line T6VS·6AL [18]. Until now, the precise physical location of this gene was not known. Owing to the low frequency of pairing and suppressed recombination between the 6VS of *H. villosa* and 6AS of *T. aestivum*, it is extremely difficult to characterize the *Stpk-V* locus through meiosis crossover and recombination.

To obtain more accurate physical mapping information of the *Stpk-V* gene and facilitate exploitation of the new resistance-related gene in the adjacent area of *Stpk-V* loci, this study used a sensitive FISH and sequential GISH in different *T. aestivum–H. villosa* alien chromosome lines, including the 6V addition line, the 6V (6A) substitution line, the 6VS/6AL translocation lines, the 6VS deletion lines and translocation lines with the small 6VS chromosome segment. The genotypes susceptible to powdery mildew, which contain the 6V chromosome segment, were also used to determine the location of the *Stpk-V* gene. The results derived from sequential TAC-FISH and GISH methods provide reliable information for the precise physical location of the *Stpk-V* gene, and are helpful for tracing the *Stpk-V* gene using the cytogenetic method in different genetic stocks.

1 Materials and methods

1.1 Plant materials

H. villosa, Triticum durum cv. 1286–*H. villosa* amphiploid (AABBVV), *T. aestivum–H. villosa* 6V addition line 06R33, 6V (6A) substitution line 06R41, 6VS/6AL translocation lines 92R90 and 92R137 and its derivative variety Nannong 9918, deletion addition line del6VS-1 lacking the distal 42% of the short arm of chromosome 6V, 6VS small segment translocation lines YC72-2 and YC138-4 with the 6VS chromosome bin at FL0.45–0.58 and 0.35–1.00, respectively, were resistant to powdery mildew. Deletion addition line del6VS-2, which lacks the distal 55% of the short arm of chromosome 6V, the 6VS small segment translocation lines HY86-1 and HY158-5, which contain the 6VS chromosome bin at FL0.00–0.45 and 0.70–1.00, respectively, were susceptible to powdery mildew. These materials were used for physical mapping of the *Pm21* locus. The *H. villosa* accession used in this study was kindly provided by the Cambridge Botanical Garden, UK. The *T. aestivum–H. villosa* 6V alien chromosome lines were developed and provided by the Cytogenetics Institute, Nanjing Agricultural University (CINAU) [13,14,19–23], where *T. durum* cv. 1286, and *T. aestivum* cv. Chinese Spring were maintained.

1.2 Molecular marker verification

Plant materials used for TAC-FISH were tested using the molecular marker CINAU15, which is specific to the *Stpk-V* gene. Genomic DNA was isolated by the CTAB method [24]. PCR was performed as described [15]. The PCR products were electrophoresed in non-denaturing 8% (w/v) polyacrylamide gels.

1.3 Chromosome preparation

Seeds were left to germinate on moist filter paper for 24 h at 22°C, kept for 24 h at 4°C followed by 24 h at 22°C. Root tips ~1.5 cm long were harvested, kept in ice-cold water for 24 h before fixation in 3∶1 (v/v) ethanol/acetic acid and then stored at −20°C. Chromosome spreads were made by squashing fixed material onto a microscope slide in a drop of 45% (v/v) acetic acid. Preparations with good chromosome morphology were immediately frozen by immersion in liquid nitrogen for 1 min. After removing the coverslip, the slide was dehydrated through a graded ethanol series (70%, 90% and 100%), then air dried.

1.4 Probe labelling

TAC15, a 30-kb positive clone containing the sequence of the *Stpk-V* gene was obtained by Sun et al. [17]. TAC15 DNA was isolated by the QIAprep Spin Miniprep Kit (QIAGEN). TAC15 and *H. villosa* genomic DNA were labelled with digoxigenin-11-dUTP (Roche Diagnostics, Germany) by standard nick translation reactions, and purified by passage through Sephadex G-50.

1.5 Sequential TAC-FISH and GISH

FISH and sequential GISH were conducted essentially as

described by Zhang et al. [25] and Dong et al. [26] with a few modifications. Chromosome-bearing slides were placed into 70% (v/v) formamide in 2×SSC and the chromosomes were denatured by heating at 88°C on a hot-plate for 3 min and immediately immersed in a graded ethanol series (70%, 90% and 100%, 5 min each) at −20°C. Total genomic DNA isolated from the leaves of *T. aestivum* cv. Chinese Spring and *H. villosa* were sheared to an average size of 100–300 bp by autoclaving at 120°C for 3 min, then used as blocking DNA at a 300-fold greater quantity than the probe. Digoxigenin-labelled probe was detected with FITC-conjugated anti-digoxigenin antibody (Roche Diagnostics, Germany). Chromosomes were counterstained with 4′,6-diamidino-phenylindole (DAPI) in an anti-fade solution (Vector). Slides were examined under a fluorescence microscope (Olympus BX61, Japan). Chromosome and FISH signal images were captured using a charge-coupled device (CCD) camera and merged using IPLab Spectrum software (Signal Analytics, USA).

The slides were then soaked in phosphate-buffered saline (pH 7.4) to aid removal of the coverslips, the DNA was denatured again in 70% (v/v) formamide at 80°C for 2 min, and incubated with labelled *H. villosa* genomic DNA for sequential GISH. Chromosome images and GISH signals in the same metaphase cells selected in FISH analysis were captured and compared with the FISH results. The fraction length (FL) of hybridization sites was calculated as the distance from the centromere to the TAC-FISH signal relative to the total length of the chromosome arm. Measurements were made on digital images using IPLab Spectrum software. More than ten chromosomes were measured for each FL calculation and standard deviations were calculated.

2 Results

2.1 Verification of materials by PCR amplification with the CINAU15 marker derived from the *Stpk-V* gene

T. aestivum–H. villosa 6V alien chromosome lines were developed in our earlier work and the PCR marker CINAU15 was designed on the basis of the sequence of the *Stpk-V* gene [13−15,19]. To further determine whether the genetic stocks used in this study conferred the *Stpk-V* gene, PCR analysis with a pair of CINAU15 primers was done using the genomic DNA of *T. aestivum* cv. Chinese Spring, *T. durum* cv. 1286, *H. villosa*, *T. durum–H. villosa* amphiploid and *T. aestivum–H. villosa* 6V alien chromosome lines. PCR products with a 902-bp band were detected in genomic DNA of these different genetic stocks containing the whole 6VS chromosome arm or the 6VS chromosome bin FL 0.00–0.58, which were *H. villosa*, *T. durum–H. villosa* amphiploid and *T. aestivum–H. villosa* 6V addition line 06R33, the 6V(6A) substitution line 06R41, the 6VS/6AL translocation line 92R90, 92R137 and its derivative variety Nannong 9918 and the deletion addition line del6VS-1 (Figure 1(a)). This 902-bp amplicon was not detected in *T. aestivum* cv. Chinese Spring, *T. durum* cv. 1286, the deletion addition line del6VS-2 that lacks the distal 55% of 6VS, the 6VS small segment translocation lines HY86-1 and HY158-5 that contain FL0.00−0.45 and 0.70−1.00 of the 6VS chromosome, respectively (Figure 1(a)). On the basis of these results of marker analysis, the *Stpk-V* gene was located on the 6VS chromosome bin (FL: 0.45−0.58) (Figure 1(b)), and the genetic stocks harbouring the *Stpk-V* gene, such as the *T. aestivum–H. villosa* 6V addition line 06R33, the 6V(6A) substitution line 06R41, the 6VS/6AL translocation lines 92R90 and 92R137, and its derivative variety Nannong 9918, and the deletion addition line del6VS-1 were suitable for subsequent cytogenetic analysis of this gene.

2.2 Physical mapping of the *Stpk-V* gene by sequential TAC-FISH and GISH in *T. aestivum–H. villosa* 6V (6A) substitution, 6V addition and 6VS/6AL translocation lines

The *T. aestivum–H. villosa* 6V(6A) substitution line 06R41 was used to determine the quantity of blocking genomic DNA in FISH with TAC15 as the probe. When the blocking DNA of *T. aestivum* cv. Chinese Spring and *H. villosa* were not used, painting-like signals were observed on a pair of 6V chromosomes and some non-specific signals were detected on chromosomes of common wheat (Figure 2(a)). The result suggested that, in addition to the *Stpk-V* gene, TAC15 contains repetitive sequence of *H. villosa*. when a 300-fold greater amount of blocking DNA of *T. aestivum* cv. Chinese Spring was added, all non-specific signals on the chromosomes of common wheat can be blocked and partial non-specific signals on the 6V chromosomes of *H. villosa* are still detectable (Figure 2(b)). However, by adding a 300-fold greater amount of blocking DNA of both *T. aestivum* cv. Chinese Spring and *H. villosa*, non-specific noise on both 6V chromosomes and the chromosomes of common wheat yielded by repetitive sequences of the TAC clone could be blocked, and clear double fluorescent signals were detected at both chromatids of a pair of chromosomes (Figure 2(c)), which were then determined to be a pair of 6V chromosomes containing the *Stpk-V* gene by a sequential GISH (Figure 2(d)). The TAC15 signals detected on the 6V chromosomes were analysed to determine the precise location of the *Stpk-V* gene, and the FL position of the *Stpk-V* locus was 0.587±0.040 (Table 1).

TAC-FISH to mitotic metaphase chromosomes of *T. aestivum–H. villosa* addition and translocation lines were used to further confirm the physical location and to facilitate statistical analysis of the position of the gene, and similar discrete hybridization signals of *Stpk-V* loci were detected (Figure 3(a), (c), (e) and (g)). Following TAC-FISH, rehybridization of the chromosome preparations with *H. villosa* genomic DNA as the probe showed that chromosomes with strong hybridization signals were 6V or 6VS (Figure 3(b), (d), (f) and (h)). The FL position of the *Stpk-V* locus

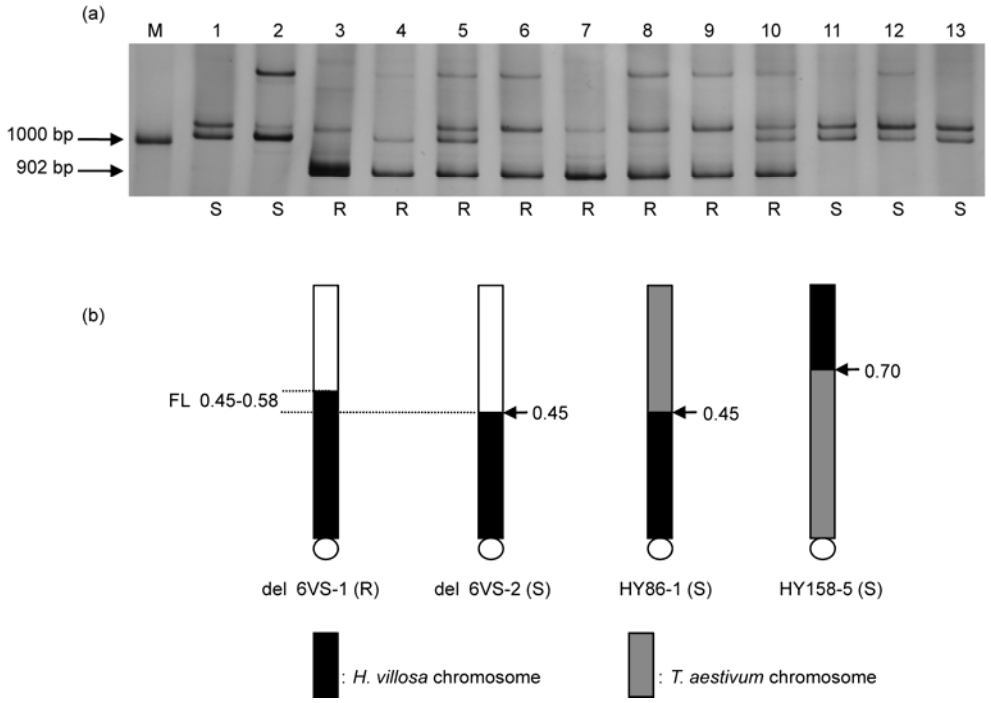

Figure 1 PCR analysis using a sequence-specific marker of the *Stpk-V* gene in different genetic stocks located the *Stpk-V* locus to the 6VS chromosome bin FL 0.45–0.58. (a) M, marker; 1, Chinese Spring; 2, *T. durum* cv. 1286; 3, *H. villosa*; 4, *T. durum* cv. 1286-*H. villosa* amphiploid; 5, *T. aestivum-H. villosa* 6V addition line 06R33; 6, *T. aestivum-H. villosa* 6V substitution line 06R41; 7, *T. aestivum-H. villosa* 6VS/6AL translocation line 92R137; 8, *T. aestivum-H. villosa* 6VS/6AL translocation line Nannong 9918; 9, *T. aestivum-H. villosa* 6VS/6AL translocation line 92R90; 10, *T. aestivum-H. villosa* 6VS deletion addition line del6VS-1; 11, *T. aestivum-H. villosa* 6VS deletion addition line del6VS-2; 12, *T. aestivum-H. villosa* 6VS small segment translocation line HY158-5; 13, *T. aestivum-H. villosa* 6VS small segment translocation line HY86-1. R: resistance to powdery mildew; S: susceptible to powdery mildew. (b) A diagram of 6VS or 6VS segment in different genetic stocks.

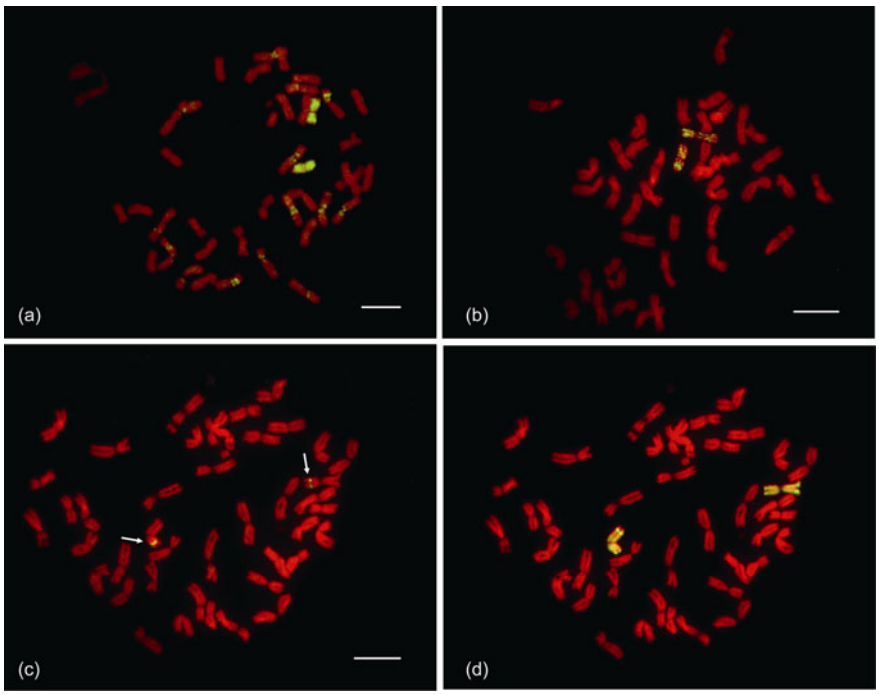

Figure 2 FISH with TAC15 as the probe on metaphase chromosomes of *T. aestivum–H. villosa* 6V substitution line 06R41. Hybridization signals were detected with FITC-anti-digoxigenin, and chromosomes were counterstained with DAPI. Chromosomes are coloured red. The scale bars represent 10 μm. (a) FISH with TAC15 as the probe on metaphase chromosomes without adding blocking DNA of Chinese Spring and *H. villosa.* (b) FISH with TAC15 as the probe on metaphase chromosomes by adding 300-fold blocking DNA of Chinese Spring. (c) FISH with TAC15 as the probe on metaphase chromosomes by adding 300-fold blocking DNA of both Chinese Spring and *H. villosa*. Arrows indicate the signals on both chromatids of chromosome 6V. (d) Sequential GISH with the genomic DNA of *H. villosa* as the probe.

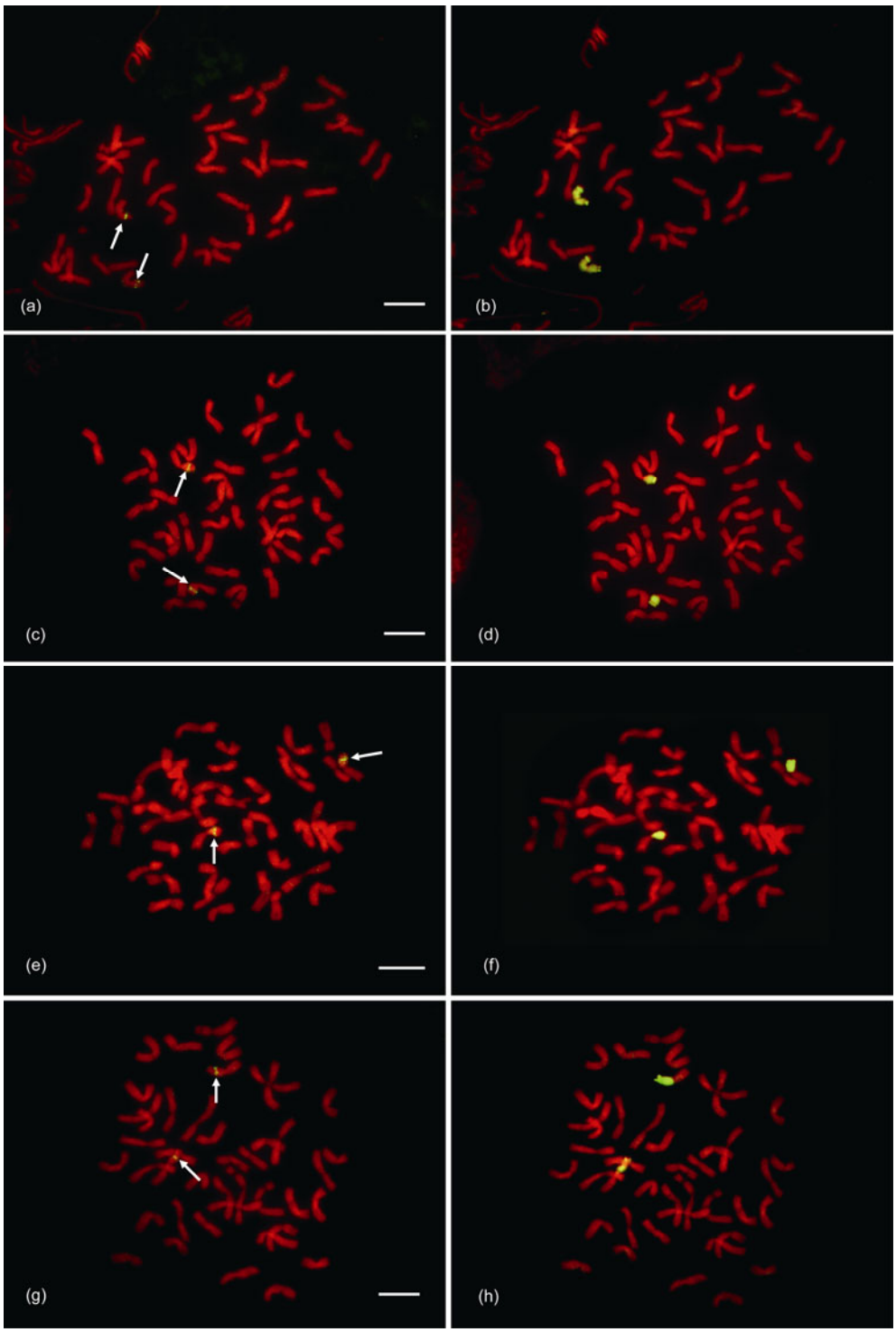

Figure 3 FISH with TAC15 as the probe (a, c, e and g) and GISH with the genomic DNA of *H. villosa* as the probe (b, d, f and h) hybridized to cells with complete mitotic metaphase chromosomes of different genetic stocks containing 6V. Hybridization signals were detected with FITC-anti-digoxigenin, and chromosomes were counterstained with DAPI. Chromosomes are coloured red. Arrows indicate the signals on both chromatids. The scale bars represent 10 μm. (a) and (b) *T. aestivum-H. villosa* 6V addition line 06R33; (c) and (d) *T. aestivum-H. villosa* 6VS/6AL translocation line 92R137; (e) and (f) *T. aestivum-H. villosa* 6VS/6AL translocation line Nannong 9918; (g) and (h) *T. aestivum-H. villosa* 6VS/6AL translocation line 92R90.

was 0.573±0.033, 0.566±0.034, 0.574±0.039 and 0.578±0.021 in *T. aestivum–H. villosa* 6V addition line 06R33, 6VS/6AL translocation line 92R137, Nannong 9918 and 92R90, respectively. In summary, the FL position of the *Stpk-V* locus was found to be 0.575±0.035 on the 6V chromosome short arm (Table 1).

Table 1 Physical location of TAC15 in different genetic stocks containing 6V chromosome

Genotype	Type	Number of chromosomes measured	FL value and standard deviation
06R33	Add 6V	13	0.573±0.033
06R41	Sub 6V	21	0.587±0.040
92R137	6VS/6AL	27	0.566±0.034
Nannong 9918	6VS/6AL	20	0.574±0.039
92R90	6VS/6AL	20	0.578±0.021
Total		101	0.575±0.035

2.3 Tracing the location of the *Stpk-V* gene by sequential TAC-FISH in *T. aestivum–H. villosa* 6V alien chromosome lines

FISH with TAC15 as the probe was used for tracing the location of the *Stpk-V* gene in different genotypes containing the 6VS chromosome segment. Discrete hybridization signals of *Stpk-V* loci were not detected in genotypes susceptible to powdery mildew, such as the alien deletion addition line del6VS-2 (FL 0.45) and the 6VS small segment translocation lines HY86-1 and HY158-5, which contain the 6VS chromosome bin at FL0.00–0.45 and 0.70–1.00, respectively (data not shown). FISH signals of the *Stpk-V* locus were detected in the deletion addition line del6VS-1 (FL 0.58) and the 6VS small translocation lines YC72-2 and YC138-4, which are resistant to powdery mildew. Figure 4(a) shows that the fluorescence signals occurring at the terminal region of a pair of chromosomes in deletion addition line del6VS-1 and chromosomes with fluorescent signals were identified as a pair of *H. villosa* chromosomes by sequential GISH with *H. villosa* genomic DNA as the probe (Figure 4(b)). Figure 4(c) and (e) show the clear doublet signals of FISH with TAC15 as the probe in the 6VS small

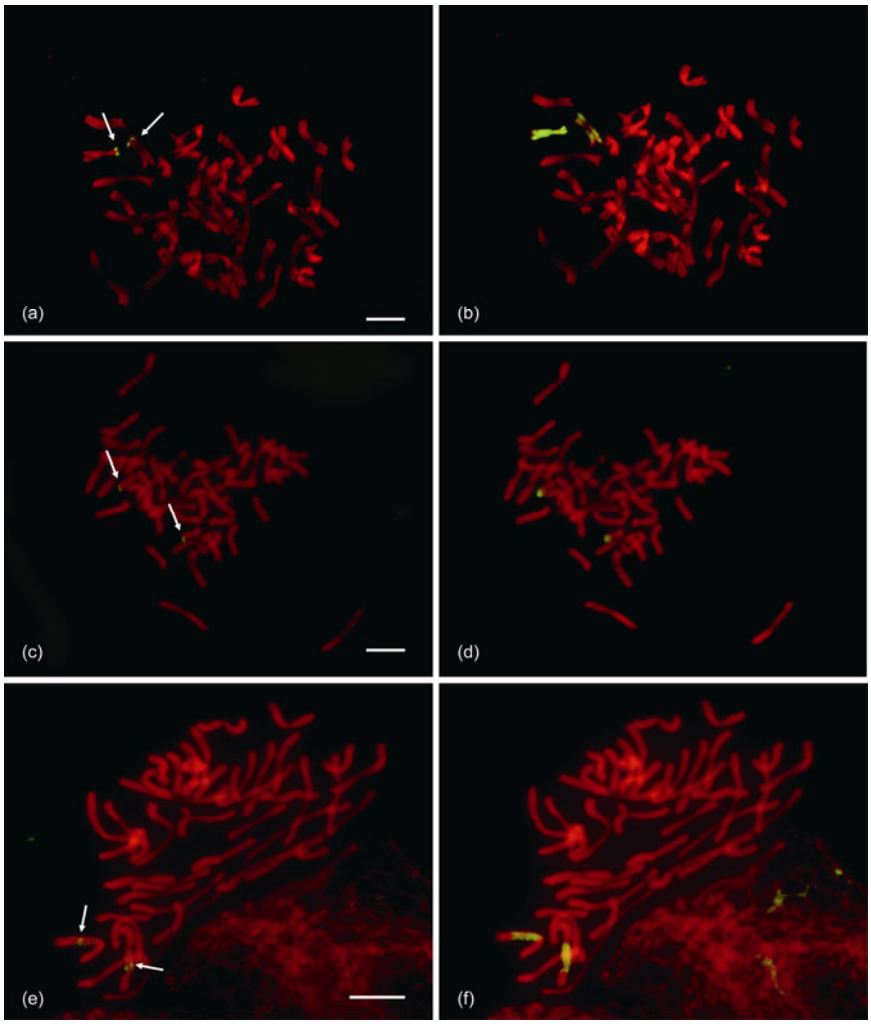

Figure 4 Sequential FISH with TAC15 as the probe (a, c and e) and GISH with the genomic DNA of *H. villosa* as the probe (b, d and f) for tracing the location of the *Stpk-V* gene in *T. aestivum–H. villosa* 6V alien chromosome lines. Hybridization signals were detected with FITC-anti-digoxigenin and chromosomes were counterstained with DAPI. Chromosomes are coloured red. Arrows indicate the signals on both chromatids. The scale bars represent 10 μm. (a) and (b) *T. aestivum-H. villosa* 6V deletion addition line del6VS-1; (c) and (d) *T. aestivum-H. villosa* 6VS small segment translocation line YC72-2; (e) and (f) *T. aestivum-H. villosa* 6VS small segment translocation line YC138-4.

segment translocation lines YC72-2 and YC138-4. Sequential GISH with the genomic DNA of *H. villosa* as the probe showed the chromosome segment carrying the *Stpk-V* gene was from the *H. villosa* chromosome (Figure 4(d) and (f)).

3 Discussion

In plants, many BACs or YACs carrying single- or low-copy sequences contain repetitive sequences that hamper detection of the single- or low-copy sequences by FISH and FISH of most plant BACs results in strong background signals from multi-site hybridization of repeated sequences. Competitive *in situ* suppression with blocking DNA, using the unlabelled *Cot*-1 fraction or sheared genomic DNA, is of significant help for mapping genes by BAC- or YAC-FISH of some plant species with relatively small genomes. In rice, the bacterial blight disease-resistance genes *Xa21* and *xa5*, the leaf blast-resistant genes *Pi-b* and *Pi-5*(*t*) and the gall midge resistance gene *Gm2* were physically mapped to metaphase chromosomes by BAC-FISH or YAC-FISH [5–9]. However, BAC-FISH for physical mapping in plants possessing large genomes, such as wheat and barley, might be questionable [3,27]. Most of the BAC clones hybridized as dispersed signals on all chromosomes and it was not easy to obtain a discrete signal even when a large excess of suppressor DNA was added. Owing to the large proportion (>80%) of repetitive sequences in genome of wheat, it is extremely difficult to generate unique locus-specific FISH signals for most wheat BACs [3,28]. In wheat, there were only a few reports about successfully mapping using BAC clones, such as the BACs screened from the *Aegilops tauschii* and *T. monococcum* could hybridized to specific chromosomes of wheat [3].

BACs or TACs with low-copy repetitive sequences were obtained in our laboratory by screening a wheat BAC or TAC library with molecular markers. Only a few BACs or TACs yielding discrete signals were identified by FISH. In this study, the quantity and type of blocking DNAs is important to suppress the background signals generated by the repetitive sequence of TAC15. When the quantity of blocking DNA was 300-fold greater compared to the amount of labelled probe TAC15, either genomic DNA from *T. aestivum* cv. Chinese Spring or from *H. villosa* could not suppress the background signals completely. Ratios for sheared genomic DNA to labelled probe ranged from 50 to 150-fold were not able to detect distinctive signals (data not shown). Specific hybridization signals generated by TAC15 are clearly distinguishable only when using a >300-fold greater amount of sheared genomic DNA of both *T. aestivum* cv. Chinese Spring and *H. villosa* compared to labelled TAC15. The signals were detected on both chromatids using different *T. aestivum–H. villosa* 6V alien chromosome lines, indicating that the signals obtained by FISH were genuine and the results of FISH mapping were reliable. After detecting >100 chromosomes of 6VS with TAC15 signals, the *Stpk-V* locus was physically located at the position of 6VS FL 0.575± 0.035.

In our earlier work, when labelled TAC15 probe was hybridized to the metaphase chromosomes of *H. villosa*, background signals produced by repetitive sequences of TAC15 were dispersed over all chromosomes and failed to suppress the background signals, even by using blocking DNA of both *T. aestivum* cv. Chinese Spring and *H. villosa*. The FISH signals of TAC15 containing *Stpk-V* loci were more easily visible with minimal interference of the V-genome in *T. aestivum–H. villosa* alien chromosome lines with a pair of V-genome chromosome than in *H. villosa* with the whole V genome. Therefore, the use of *T. aestivum–H. villosa* 6V alien chromosome lines and genomic DNA of both *T. aestivum* cv. Chinese Spring and *H. villosa* for blocking are important for mapping the *Stpk-V* gene. The gene physical mapping strategies used in this study will be helpful for tracing alien genes from wild species in *T. aestivum*.

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